Journal of Medical Virology 60:86–90 (2000)

Detection of Neurotropic Viruses Circulating in Tuscany: The Incisive Role of Toscana Virus

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Acute meningitis is perhaps the most frequent among central nervous system infections. We report a study considering 277 cases of meningitis hospitalized in the southern Tuscany area (Italy) during the period from 1995 to 1998 investigated by tissue culture and polymerase chain reaction (PCR) methods. The cytochemical analysis of the cerebrospinal fluid samples suggested the diagnosis of aseptic meningitis, recognized as viral meningitis in 104 cases by detection of viral DNA or RNA. The results collected by tissue culture technique, available for 95 clinical samples, reported a positive isolation for only 12 cases. The viruses identified in the neurological infection were Toscana virus (81%), enterovirus (12%), mumps virus (3%), measles virus (1%), and herpes virus type 1 (3%). These data demonstrate the incisive role of the RNA viruses as the cause of meningitis, and overall the relevance of Toscana virus. ***J. Med. Virol. 60:86–90, 2000.***

KEY WORDS: meningitis; polymerase chain reaction; viral RNA; cerebrospinal fluid

INTRODUCTION

Acute meningitis is a neurological disease that requires early detection, rapid identification of the etiologic agent (bacterial, viral, or other), and rapid treatment [McGee and Kaiser, 1990]. The occurrence of cases of infectious meningitis involves the previous colonization of distant anatomic sites by a wide variety of microorganisms, attachment to respiratory mucosal cells, invasion and survival of the microorganism in the circulatory system, penetration into the cerebrospinal fluid (CSF), and a specific neurotropism of the microorganism. In viral meningitis the etiologic agents most often reach the nervous system through the bloodstream or through the neural pathways. The hematogenous route is used by most viruses causing meningitis after they undergo multiplication at the site of entry or in regional lymph nodes, draining the entry site [Cassady and Whitley, 1997]. In a case of subacute presentation, early diagnosis is fundamental. The most serious neurological signs are rare in viral meningitis, and most cases present a short and benign course without therapy. The predominant causative or associated viral agents of meningitis are enteroviruses, flaviviruses, bunyaviruses, mumps virus, measles virus, herpes simplex virus types 1 and 2, herpes zoster virus, adenovirus, cytomegalovirus, lymphocytic choriomeningitis virus, and Epstein-Barr virus [Deibel et al., 1979; Bergstrom et al., 1990; Soeur et al., 1991; Shoji et al., 1992; Kaji and Shoji, 1995; Kudelova et al., 1995; Cusi et al., 1996; Luzi et al., 1997; Valassina et al., 1996; Rousseau et al., 1997; Gorgievski-Hrisoho et al., 1998; Rotbart et al., 1998; Tsai et al., 1998]. Standard diagnostic procedures, such as viral culture or serology, are often insufficient for the circumstantial or retrospective identification of a viral infection of the central nervous system [Chonmaitree et al., 1989]. Molecular techniques appear to be valid support useful for the accurate detection of the viral agent in the CSF, considering the rapidity of execution and their sensitivity [Hosoya et al., 1998; Read and Kurtz, 1999]. We applied the molecular techniques of polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) for the detection of viruses as etiologic agents in 277 cases of meningitis which occurred in Siena (Italy) during the period from 1995 to 1998 in order to evaluate the viral agent that is the most frequent cause of meningitis in this geographic area.

MATERIALS AND METHODS

During the 4-year period from 1995 to 1998, 277 patients (3–78 years old; mean age 29.2 years; 179 males and 98 females) hospitalized with meningitis were enrolled in this study. Immunocompromised patients, such HIV-infected subjects, patients with leukemia, and transplant recipients, were excluded from the study. The seasonal distribution of the hospitalized population was 41 during the December–February period, 26 in March–May, 156 in June–August, and 54 in September–November. The patients showed clinical

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Accepted 29 June 1999

symptoms of meningitis: fever, headache, vomiting, ocular pain, neck rigidity. The CSF specimens were drawn from the patients at the time of hospitalization, aliquoted, and analyzed for chemical and cytological criteria (white blood cell count, protein and glucose concentrations). Standard isolation procedures were adopted to detect bacterial and mycological pathogens. Vero cells (ATCC CCL 81) were used for virus isolation when a sufficient volume of CSF was obtained. The cells were maintained in Eagle's minimal essential medium (Life Technologies, Milan, Italy) and supplemented with 5% fetal calf serum (Life Technologies) and penicillin (200 U/ml)-streptomycin (200 mg/ml) (Sigma Co., St. Louis, MO). All the CSF specimens were inoculated in Vero cells and then incubated at 37°C in Eagle's minimal essential medium until a cytopathic effect appeared. The negative cell cultures were maintained for 14 days after the supernatant was harvested and blind passaged on cells. An aliquot of CSF was subjected to DNA and RNA extraction according to the procedures described in the High Pure Viral Nucleic Acid kit (Boehringer Mannheim, Milan, Italy). The extracted nucleic acid was then tested to verify its amplificability by detection of the ubiquitous β-globin human gene and the messenger of the glyceraldehyde-3-phosphate-dehydrogenase human gene as described previously [Ercolani et al., 1988; Valassina et al., 1995]. The nucleic acid samples were investigated in nested PCR for adenovirus (ADV) [Hierholzer et al., 1993], herpes simplex virus types 1 and 2 (HSV-1, HSV-2) [Aurelius et al., 1991, 1993], cytomegalovirus (CMV), herpes-zoster virus (HZV) [Puchhammer et al., 1991], Epstein-Barr virus (EBV) [Oosterveer et al., 1993], and nested RT-PCR for enteroviruses [Kammerer et al., 1994], Toscana virus [Valassina et al., 1996], measles virus [Godec et al., 1990], mumps virus [Cusi et al., 1996], and tick-borne encephalitis virus (TBE) using the primers listed in Table I. The specific primers for CMV and TBE virus were selected and tested on positive samples in our laboratory. The protocol of amplification was performed at 94°C for 1 min, 52°C for 45 sec, 72°C for 1 min for ADV, CMV, HSV-1, HSV-2, EBV, HZV, and enteroviruses. The annealing temperature for measles virus, Toscana virus, TBE, and mumps virus was 56°C. Annealing temperature was increased to 54°C and 58°C, respectively, in nested PCR assays. All the PCR and RT-PCR procedures were carried out according to the recommended guidelines [Innis and Gelfand, 1990], including three negative controls and one positive control in each assay starting with the nucleic acid extraction step [Hoad and Schulz, 1991].

RESULTS

Chemical and cytological analyses showed a CSF cell count of $>10^3/mm^3$, protein amounts of >50 mg/dl, and a glucose concentration of >60%. These chemical parameters are commonly referred to as aseptic meningitis [Retzan, 1985; McGee and Kaiser, 1990], and supported by the failure of bacterial and mycological isolation in CSF samples. The amount of CSF was sufficient for viral isolation on Vero cells in only 95 cases (11 collected in December–February, 8 in March–May, 62 in June–August, and 14 in September–November). Among these, 12 specimens drawn from the 62 patients with meningitis during the summer period were positive for cell culture. All the isolated viruses showed a lytic cytopathic effect on cell culture and they were all positive for hemoadsorption with goose erythrocytes. No positive results were found by the isolation method for the detection of other neurotropic viruses. The negative cell cultures were maintained for 14 days after the supernatant was harvested and blind passaged on cells.

All 277 samples that had previously proved suitable for PCR and RT-PCR were tested for the detection of neurotropic DNA and RNA viruses by molecular assays. We detected neurotropic RNA viruses in 101/277 cases (85 Toscana virus, 12 enteroviruses, 3 mumps virus, and 1 measles virus) by RT-PCR. HSV-1 was identified by PCR in 3/277 cases. We were unsuccessful in detecting any neurotropic viruses in the remaining 173 patients (62.4%) using the specific primers available in our laboratory. The PCR positive results obtained are shown in Figure 1. All samples that were positive by cell culture were identified as Toscana virus by RT-PCR. No sample that was negative by PCR or RT-PCR was positive by tissue culture isolation. The seasonal distribution of meningitis cases was also analyzed. Considering the total population hospitalized with meningitis during the 1995–1998 period, 156 cases (56.3%) occurred during the summer period (June–September) and 121 cases (43.7%) occurred in the October–May period. Among the 156 cases, 91 were diagnosed: of these, 83 were positive for Toscana virus, 6 were positive for enteroviruses, and 2 were positive for mumps virus. Among the remaining 121 cases (43.7%) of meningitis in the October–May period, it was possible to identify the etiologic agent in only 13 cases (10.3%): 6 of these were positive for enterovirus, 2 for Toscana virus (in October), 1 for mumps virus, 1 for measles virus, and 3 for HSV-1. No sample was positive for TBE virus, HSV-2 virus, CMV, HZV, EBV, or ADV.

DISCUSSION

The viral agents responsible for the infection of the central nervous system are often difficult to identify with conventional isolation methods mostly due to a low amount of the viral charge in the CSF sample at the time of the clinical manifestation. The existing standard techniques are often insensitive and slow, or they require invasive samples [Lindeman et al., 1974; Chonmaitree et al., 1989]. The serological analysis usually only provides the retrospective evidence of viral neurological infection, which is not useful for the rapid treatment of the acute severe disease. IgM capture ELISA performed on sera and CSFs has been shown to be useful in early diagnosis of neurological infections [Heyman, 1997; Jones et al., 1999], but sometimes the

TABLE I. List of Specific Primers Used for Detection of DNA and RNA of Neurotropic Viruses by Nested PCR*

Primer	Virus	Gene	Position
MF 1	Measles	Fusion	1066–1087
MF 2	Measles	Fusion	1496–1517
MF 3	Measles	Fusion	1166–1188
MF 4	Measles	Fusion	1377–1391
F1	Mumps	Fusion	64–84
F2	Mumps	Fusion	748–768
F16S	Mumps	Fusion	332–352
F17S	Mumps	Fusion	548–569
ENT 3	Enterovirus	5′ constant region	67–83
ENT 4	Enterovirus	5′ constant region	545–561
ENT 5	Enterovirus	5′ constant region	166–182
ENT 6	Enterovirus	5′ constant region	447–463
TV 1	Toscana	N (small segment)	267–288
TV 2	Toscana	N (small segment)	669–688
TV 3	Toscana	N (small segment)	308–330
TV 4	Toscana	N (small segment)	595–617
TBE 1	TBE	Envelope glycoprotein	187–207
TBE 2	TBE	Envelope glycoprotein	577–595
TBE 3	TBE	Envelope glycoprotein	264–284
TBE 4	TBE	Envelope glycoprotein	456–477
HSV 1A	HSV-1	gD	26–43
HSV 1B	HSV-1	gD	218–239
HSV 1C	HSV-1	gD	51–71
HSV 1D	HSV-1	gD	169–188
HSV 2A	HSV-2	gG2	363–380
HSV 2B	HSV-2	gG2	525–546
HSV 2C	HSV-2	gG2	422–440
HSV 2D	HSV-2	gG2	506–522
CMV A	CMV	IE1	2181–2201
CMV B	CMV	IE1	2633–2653
CMV C	CMV	IE1	2242–2261
CMV D	CMV	IE1	2406–2425
ADV A	Adenovirus	AD2	2728–2746
ADV B	Adenovirus	AD2	2866–2885
ADV C	Adenovirus	AD2	2750–2771
ADV D	Adenovirus	AD2	2843–2860
EBV A	EBV	BKRF1	109332–109351
EBV B	EBV	BKRF1	109609–109628
EBV C	EBV	BKRF1	109353–109371
EBV D	EBV	BKRF1	109542–109561
HZV A	Varicella-Zoster	Xba M region	3377–3396
HZV B	Varicella-Zoster	Xba M region	3643–3624
HZV C	Varicella-Zoster	Xba M region	3408–3428
HZV D	Varicella-Zoster	Xba M region	3601–3621

*TBE, tick-borne encephalitis virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; CMV, cytomegalovirus; EBV, Epstein-Barr virus.

availability of such tests is not possible for all of the viruses (e.g., enteroviruses). Therefore, the use of PCR for the detection of viral DNA or RNA is a quick and sensitive molecular technique that can be very useful for the diagnosis of viral meningitis [Hosoya et al., 1998; Read and Kurtz, 1999]. However, it is necessary to improve the clinical usefulness of the PCR due to the lack of a comparable gold-standard test [Jeffery et al., 1997].

We collected 277 cases of subacute meningitis, excluding immunocompromised patients, with a consistent suspicion of viral etiology, considering the chemical and cytological data from the CSF specimen and the seasonal and geographical distribution. In order to obtain epidemiological data referring to the circulation of the neurotropic viruses causing meningitis, we observed that, during the 1995–1998 period in Tuscany, the number of cases of meningitis presented a significant increase during the summer season (156/277), with a peak during the month of August (79/156). These data agree with epidemiological studies that assess the viral etiology of meningitis in July, August, and September in younger and adult patients [McGee and Kaiser, 1990]. The principal virus causing meningitis in Tuscany appears to be the Toscana virus, a bunyavirus transmitted by the sandfly vector [Calisher et al., 1987; Nicoletti et al., 1991; Valassina et al., 1996]. Analysis of the diagnostic data collected by molecular analysis revealed the Toscana virus in 83/156 cases. Among these, 44 occurred in August when the activity of the insect vector is more intensive [Tesh, 1988]. We used the PCR technique for the clinical diagnosis of a wide range of viral pathogens and compared it with the isolation method on Vero cell cultures

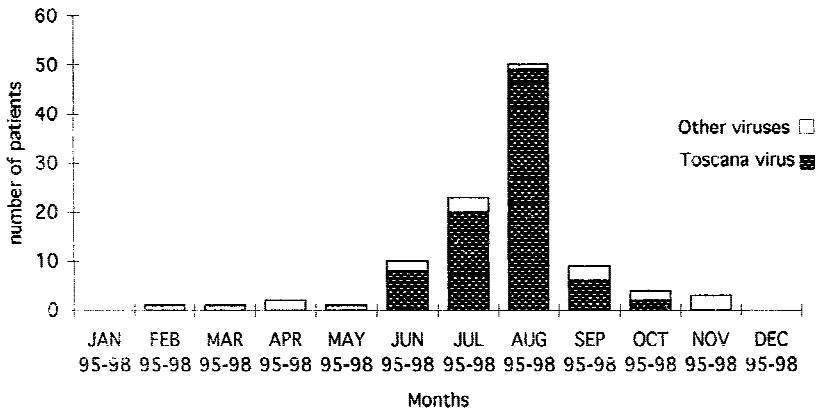

Fig. 1. Monthly distribution of PCR positive cases of meningitis during the 1995–1998 period in southern Tuscany. Prevalence of Toscana virus infection.

which are permissive to many neurotropic viruses. Virus isolation on tissue culture of the 95 CSF specimens appeared to have a low sensitivity for detecting the etiologic agent. In fact, only 12 samples that were already identified as Toscana virus were isolated on cell culture. This could be due to the small amount (200 μl) of the clinical specimen that was used for the cultivation method, and that a low viral charge was present in the CSF specimen. Two other RNA viruses detected by RT-PCR, the enterovirus and the mumps virus, were responsible for meningitis during the summer, however, we failed to identify DNA viruses in patients with meningitis during this same period. If the particular data concerning the Toscana virus infection are ruled out, the viral DNA or RNA has been identified in 9.8% of cases (19/192), which demonstrates an effective utility of this technique [Jeffery et al., 1997]. This result appears to be more significant if the data regarding the diagnosis of the Toscana virus infection are included. In fact, the diagnosis of viral meningitis was possible in 104/277 (37.5%) cases. The tissue culture results are less sensitive than those of PCR assay: 12/95 (12.6%) samples were positive only for Toscana virus. It was interesting to note that the most frequent etiologic agents of viral meningitis were RNA viruses, such as the enterovirus, and particularly the Toscana virus, which was responsible for 54.4% of the cases of meningitis in the summer period. It would be interesting to compare our results with other epidemiological data regarding other regions of the Mediterranean area where the Toscana virus is a newly emerging virus that causes meningitis during the summer. It would be helpful if all the laboratories could also test CSF samples drawn from patients with meningitis for the presence of Toscana virus RNA by using RT-PCR. The PCR approach appears to be cost-effective and it provides a rapid clinical diagnosis. The flexibility of the molecular system can also provide the possibility of a multiplex protocol [Casas et al., 1999] for detecting most of the viral agents in central nervous system diseases at the same time, with the advantage of a more rapid diagnostic response by using a relatively small aliquot of the sample.

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